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Abstract: The asymmetric outer membrane (OM) of Gram-negative bacteria contains lipopolysaccharide (LPS) in the outer leaflet and phospholipid in the inner leaflet. During OM biogenesis, LPS is transported from the periplasm into the outer leaflet by a complex comprising the OM proteins LptD and LptE. Recently, a new family of macrocyclic peptidomimetic antibiotics that interact with LptD of the opportunistic human pathogen Pseudomonas aeruginosa was discovered. Here we provide evidence that the peptidomimetics inhibit the LPS transport function of LptD. One approach to monitor LPS transport involved studies of lipid A modifications. Some modifications occur only in the inner membrane while others occur only in the OM, and thus provide markers for LPS transport within the bacterial envelope. We prepared a conditional lptD mutant of P. aeruginosa PAO1 that allowed control of lptD expression from the rhamnose promoter. With this mutant, the effects caused by the antibiotic on the wild-type strain were compared with those caused by depleting LptD in the mutant strain. When LptD was depleted in the mutant, electron microscopy revealed accumulation of membrane-like material within cells and OM blebbing; this mirrored similar effects in the wild-type strain caused by the antibiotic. Moreover, the bacterium responded to the antibiotic, and to depletion of LptD, by introducing the same lipid A modifications, consistent with inhibition by the antibiotic of LptD-mediated LPS transport. This conclusion was further supported by monitoring the radiolabelling of LPS from [^{1} C]acetate, and by fractionation of IM and OM components. Overall, the results provide support for a mechanism of action for the peptidomimetic antibiotics that involves inhibition of LPS transport to the cell surface.

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Inhibition of Lipopolysaccharide Transport to the Outer Membrane in *Pseudomonas aeruginosa* by Peptidomimetic Antibiotics

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The asymmetric outer membrane (OM) of Gram-negative bacteria contains lipopolysaccharide (LPS) in the outer leaflet and phospholipid in the inner leaflet. During OM biogenesis, LPS is transported from the periplasm into the outer leaflet by a complex comprising the OM proteins LptD and LptE. Recently, a new family of macrocyclic peptidomimetic antibiotics that interact with LptD of the opportunistic human pathogen *Pseudomonas aeruginosa* was discovered. Here we provide evidence that the peptidomimetics inhibit the LPS transport function of LptD. One approach to monitor LPS transport involved studies of lipid A modifications. Some modifications occur only in the inner membrane while others occur only in the OM, and thus provide markers for LPS transport within the bacterial envelope. We prepared a conditional *lptD* mutant of *P. aeruginosa* PAO1 that allowed control of *lptD* expression from the rhamnose promoter. With this mutant, the effects caused by the antibiotic on the wild-type strain were compared with those caused by depleting LptD in the mutant strain. When LptD was depleted in the mutant, electron microscopy revealed accumulation of membrane-like material within cells and OM blebbing; this mirrored similar effects in the wild-type strain caused by the antibiotic. Moreover, the bacterium responded to the antibiotic, and to depletion of LptD, by introducing the same lipid A modifications, consistent with inhibition by the antibiotic of LptD-mediated LPS transport. This conclusion was further supported by monitoring the radiolabelling of LPS from [^{14}C]acetate, and by fractionation of IM and OM components. Overall, the results provide support for a mechanism of action for the peptidomimetic antibiotics that involves inhibition of LPS transport to the cell surface.

Introduction

A family of macrocyclic peptidomimetic antibiotics (e.g., L27–11, Figure 1 A) was shown recently to exhibit potent antimicrobial activity against Gram-negative *Pseudomonas* sp., including the problematic human pathogen *Pseudomonas aeruginosa*. A photoprobe derived from L27–11 was shown by photoaffinity labelling to bind to the outer membrane (OM) protein LptD in *P. aeruginosa*.[1] LptD fulfils an essential role in OM biogenesis in many Gram-negative bacteria, where it functions in a complex with the lipoprotein LptE to transport lipopolysaccharide (LPS) from the periplasm to the outer cell surface (Figure 1 B).[2] LptD is predicted from bioinformatic analysis to contain a C-terminal β-barrel domain of about 600 residues embedded in the OM and an N-terminal domain of about 300 residues that sits on the periplasmic side of the OM.

Resistance to many currently used antibiotics is a growing problem with *P. aeruginosa*, caused in part by the permeability barrier imposed by the asymmetric outer cell membrane, which comprises LPS in the outer leaflet and phospholipids in the inner.[3] In this work, we set out to investigate whether the mechanism of action of L27–11 involves inhibition of LptD-mediated LPS transport to the outer cell surface in *P. aeruginosa*. Presently, it cannot be excluded that LptD transports the antibiotic across the OM, so that it can interact with an as yet unknown target within the cell. Ample evidence exists that β-barrel OM proteins in Gram-negative bacteria can aid the cellular uptake of peptide antibiotics, such as microcin J25 and the colicins.[4] If this hypothesis is correct, however, there should be no major effects by the antibiotic on LPS transport to the cell surface. However, changes in *Pseudomonas* cell morphology caused by L27–11 were detected by electron microscopy (EM) in earlier work, which is consistent with a mechanism of action that impacts on cell wall biogenesis.[1] So far, no other molecules have been described that interact with LptD.

For this reason we considered it important to search for additional evidence that antibiotic L27–11 inhibits LPS transport

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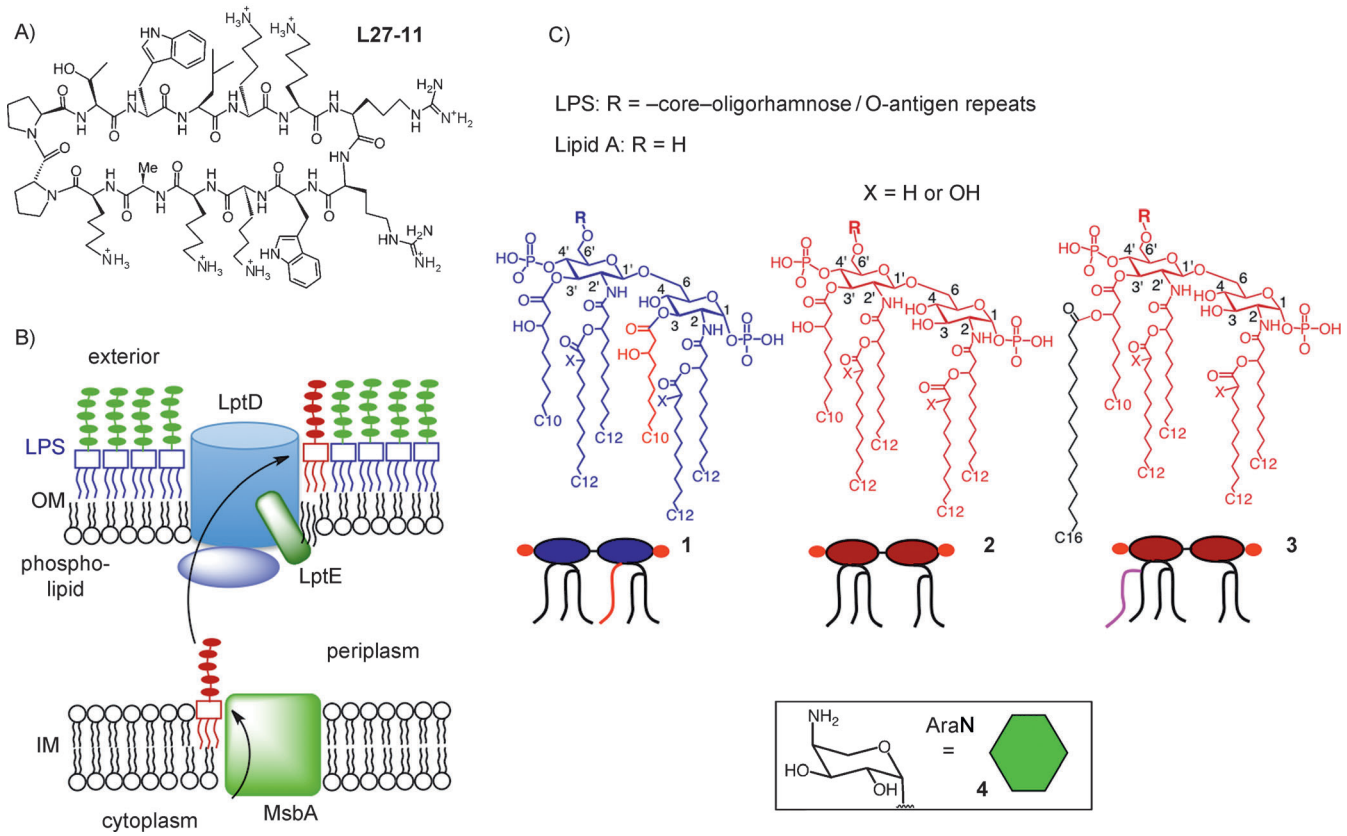

Figure 1. A) Structure of antibiotic L27–11. B) LPS transport in *Pseudomonas aeruginosa*. LPS assembly occurs in the cytoplasm and on the IM. Key tailoring reactions occur either on the IM or the OM (see text). C) The conserved lipid A portion of LPS from *P. aeruginosa*. Structures: **1**) before modification by PagL; **2**) after the action of PagL; and **3**) after the action of PagP. AraN sugar (**4**) can be added to the 1- and 4′-phosphate groups of lipid A.

to the OM in *P. aeruginosa*. Currently, no in vitro assay exists for LPS transport by the purified LptD/E complex. Instead, we have exploited methods used in earlier functional studies of LptD/E in Gram-negative bacteria; these involve monitoring lipid A modifications that occur en-route to the OM.[2c,5]

LPS from *P. aeruginosa* contains lipid A (Figure 1C) linked to a carbohydrate core that is in turn linked either to oligorhamnose or to a highly immunogenic O-antigen polysaccharide. The core–lipid A portion of LPS in most Gram-negative bacteria is biosynthesised on the cytoplasmic side of the inner membrane (IM). The core–lipid A is then flipped to the outer surface of the IM by the ABC transporter MsbA, where more oligosaccharides (oligorhamnose or the O-antigen) are added. However, before transport across the periplasm to the OM, two α-hydroxylations of fatty acyl chains in lipid A can occur in *P. aeruginosa* (shown as X = H or OH in Figure 1C), catalysed by two different Fe^{2+}/α-ketoglutarate-dependent hydroxylases (LpxO family of hydroxylases: genes PA4512 and PA1936).[5b] Only a single *lpxO* gene is present in *Salmonella* spp., and this hydroxylase has been characterised biochemically in vitro.[6] However, no *lpxO* genes are present in *Escherichia coli*. LPS isolated from laboratory strains of *P. aeruginosa* comprises mostly mono- and some di-hydroxylated forms (see below).

Further tailoring modifications occur to lipid A after incorporation of LPS into the outer leaflet of the OM. LPS isolated from laboratory strains of *P. aeruginosa* typically comprise a penta-acyl form (**2**),[7] which lacks a 3-O-fatty acyl chain (compare with **1**, Figure 1C). The 3-O-deacylation step is catalysed by the eight-stranded β-barrel enzyme PagL, which is integrated in the OM. The crystal structure of PagL from *P. aeruginosa* reveals an active site with a Ser-His-Glu catalytic triad close to the external OM surface.[8] Furthermore, and typically as part of a membrane stress response to cationic antimicrobial peptides or divalent metal ion limitation, a further modification can occur to lipid A, catalysed by the β-barrel OM enzyme PagP. PagP catalyses transfer of a palmitoyl group from a phospholipid to the β-OH of the 3′ O-acyl fatty acyl group, which increases lipid A acylation (as in the hexa-acyl form **3**, Figure 1C).[9] Solution and X-ray structures of the homologous PagP from *E. coli* reveal an eight-stranded β-barrel, and an active site again close to the extracellular side of the OM.[10]

Another lipid A modification that can occur on the IM in *P. aeruginosa*, for example as part of a resistance mechanism to cationic antimicrobial peptides, is addition of the sugar 4-amino-4-deoxy-L-arabinose (AraN, **4**) to one or both of the 1 and 4′-phosphate groups (Figure 1C).[11] *P. aeruginosa* isolates from cystic fibrosis patients were shown to possess LPS modified by addition of one or two AraN sugars to the lipid A core.[7a,d]

As these lipid A-modifying enzymes are located either in the IM or OM, the corresponding lipid A modifications have often been used as reporters for LPS trafficking within the bacterial envelope.[2b,c,5b] We make use of this here in an ESI-MS analysis of lipid A isolated from *P. aeruginosa* exposed to L27–11. We also prepared a conditional *P. aeruginosa* PAO1 *lptD* mutant, in which expression of the *lptD* gene is controlled by the level of rhamnose in the medium. With this mutant the effects of depleting *lptD* can be compared with the effects of the antibiotic on the wild-type strain. By these and other methods, our results support the hypothesis that the mode of action of the antibiotic L27–11 involves inhibition of LptD-mediated LPS transport to the cell surface. This most likely leads to major perturbation of OM structure and morphology, as was detected by EM in an earlier work.[1]

Results

Construction of an *lptD* conditional mutant.

In *E. coli*, *lptD* forms an operon with *surA* and *pdxA* (*lptD-surA-pdxA*) that is transcribed from a σ^E-dependent promoter.[2a] A similar organisation of overlapping *lptD-surA-pdxA* genes is found in the *P. aeruginosa* chromosome (Figure 2A). A conditional mutant was obtained by replacing the native *lptD* promoter in the chromosome with the rhamnose-inducible promoter P_{rhaB} (see Figure 2B and the Experimental Section).[12] The conditional mutant, PAO1 P_{rhaB}*lptD*, grew only in medium containing rhamnose. In liquid LB medium with 0.5% glucose, growth was strong when rhamnose ($\geq$0.01% (*w*/*v*)) was present, but was weak with 0.001% rhamnose (Figure S2). To assess whether or not the cell envelope was compromised, fluorescent staining was carried out with Syto9 and propidium iodide (LIVE/DEAD BacLight viability kit; Molecular Probes/Invitrogen). Fluorescence microscopy of PAO1 P_{rhaB}*lptD* cells grown under permissive conditions ($\geq$0.01% (*w*/*v*) rhamnose) revealed green staining comparable to the PAO1 strain, whereas under non-permissive conditions (0.001% (*w*/*v*) rhamnose), most cells were stained red, thus suggesting a loss of viability and a compromised cell envelope (Figure S3).

To investigate whether polar effects influence growth, the conditional mutant was complemented in *trans* with different regions of the *lptD-surA-pdxA* operon (see the Supporting Information), by using the *E. coli–Pseudomonas* shuttle plasmid pUCP-Nde.[13] Induction of the plasmid-borne *lptD* gene alone with IPTG restored growth of the mutant under non-permissive conditions (0.001% (*w*/*v*) rhamnose) to 70% of normal levels, and for cells containing both *lptD* and *surA* genes, growth returned to 90% of normal levels (Figure 2C). To compensate for possible effects caused by loss of *pdxA*, growth media with or without added vitamin B_6 were used, but no significant effect on growth under any conditions was observed. These experiments confirmed that *lptD* is an essential gene for the growth of *P. aeruginosa* PAO1.

To examine whether OM defects occur in the P_{rhaB}*lptD* mutant, growth on solid medium was monitored in the presence of 0.5% SDS and 0.5 mM EDTA, after introduction of either *lptD* alone or the overlapping *lptD-surA* genes (Figure S4). Unlike the wild-type (wt) strain, the conditional mutant was unable to grow on agar containing high rhamnose (0.1% (*w*/*v*)) when SDS/EDTA were present, thus indicating OM susceptibility to the disruptive effects of detergent/EDTA. Complementation with *lptD* alone restored growth of the mutant to wt levels on LB medium without rhamnose (as expected), but again not when SDS/EDTA was present. However, complementation with *lptD-surA* genes allowed growth on LB agar plates without rhamnose (and also in the presence of SDS/EDTA), thus indicating that both genes are required to provide resistance to detergent (Figure S4). This result is consistent with an important function for SurA in the assembly of LptD/E in *P. aeruginosa*.

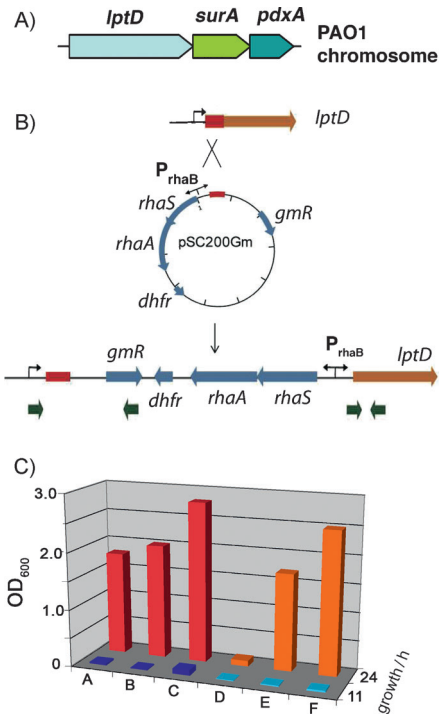

Figure 2. A) Overlapping *lptD*, *surA* and *pdxA* probably form an operon in *P. aeruginosa*. B) Construction of the *lptD* conditional mutant. After integration of the plasmid into the chromosome, *lptD* is under control of a rhamnose promoter (P_{rhaB}). Red depicts the promoter region of *lptD*; green arrows show the locations of primers used for PCR to confirm the site of integration. C) Complementation of the mutant. OD_{600} values at two time points (11 and 24 h) during growth of the mutant containing: (A and D) empty vector pUCP-Nde, (B and E) vector with *lptD*, and (C and F) vector with *lptD*+*surA*. (A–C: high, 0.1% (*w*/*v*), rhamnose, D–F: low, 0.001% (*w*/*v*) rhamnose, mutants with *lptD* or *lptD*+*surA* showed strong growth after 24 h).

Analysis of lipid A from antibiotic-treated *P. aeruginosa*

Lipid A derived from *P. aeruginosa* PAO1 LPS was analysed by high-resolution (HR) ESI-MS and by HR-ESI-MS/MS methods in negative-ion mode. The results are shown in Figure 3 and in Table S1 and S2. Normal growth in Mueller–Hinton (MH) medium afforded lipid A: ESI-MS *m*/*z* 714.4 and 722.4 for the

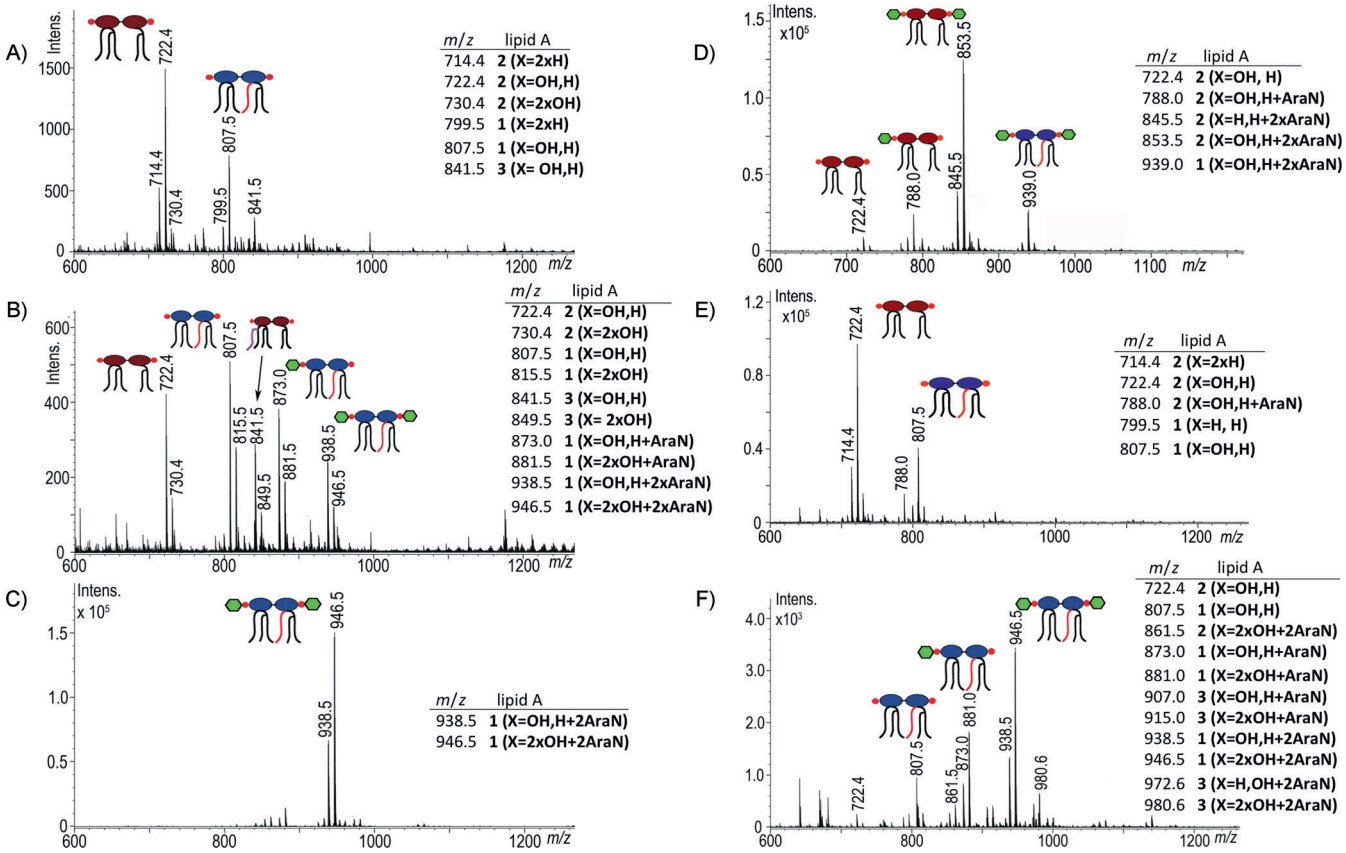

Figure 3. Negative mode ESI-MS spectra showing doubly-charged ions from lipid A species (see Table S1) isolated from *P. aeruginosa* PAO1; A) grown in MH broth; B) grown with antibiotic L27–11 for 1.25 h; C) grown with L27–11 for 6 h; D) grown with enantiomer of L27–11. PAO1 P_{rhaB}*lptD* grown with E) high rhamnose, and F) low rhamnose. The assignments of key peaks are derived from HR-ESI-MS and HR-ESI-MS/MS measurements (see the Supporting Information). Cartoons as shown in Figure 1.

doubly charged $[M-2H]^{2-}$ ion (calcd mass 1430.9 and 1446.9) corresponding to penta-acyl form **2** (non- and monohydroxylated, X=H,H and X=H,OH Figure 1 C); smaller peaks at *m/z* 799.5/807.5 corresponding to the hexa-acyl form **1** (non- and mono-hydroxylated); and a small peak at *m/z* 841.5 corresponding to the hexa-acyl form **3** (mono-hydroxylated; Figure 3, spectrum A). The structure assignments and relative proportions were confirmed by HR-ESI-MS (Table S1) and by HR-ESI-MS/MS measurements (Table S2).

This pattern of lipid A molecules changed rapidly when antibiotic L27–11 was added to liquid culture in MH broth. After 1.25 h, new peaks were seen (Figure 3, spectrum B) because of accumulation of **1** (*m/z* 807.5/815.5), as well as **1** containing one or two AraN sugars (*m/z* 873.0/881.5/938.5/946.5), and there was an increased proportion of the hexa-acyl form **3** (*m/z* 841.5/849.5). Moreover, the ratio of di- to mono-hydroxylated lipid A species increased because of a higher extent of dihydroxylation in the IM (Table S1). After 6 h growth in medium containing L27–11, the hexa-acyl form **1**, which was mainly dihydroxylated with 2 AraN sugars (*m/z* 938.5/946.5), was predominant (Figure 3, spectrum C). These results demonstrate rapid accumulation of LPS forms in antibiotic-treated cells that have not been processed by PagL.

In contrast, addition of the enantiomer of L27–11 (ent-L27–11) to growth medium did not inhibit bacterial growth and caused only a slow change in the lipid A profile: after 6 h, lipid A **2** containing two AraN sugar units predominated (Figure 3, spectrum D and Table S1), and this had been processed by PagL. This result also shows that the presence of AraN sugars does not prevent processing by PagL, in agreement with earlier studies.[7d]

Analysis of lipid A from the PAO1 P_{rhaB}*lptD* mutant

To test whether down-regulation of *lptD* produces effects on lipid A modification that are similar to those induced by the antibiotic, lipid A was prepared from the conditional PAO1 P_{rhaB}*lptD* mutant grown with high rhamnose and analysed by ESI-MS. The MS spectrum (Figure 3, spectrum E and Table S1) showed a major peak at *m/z* 722.4 (lipid A species **2**) and a smaller peak at *m/z* 807.5 (species **1**), similar to that of spectrum A for wt PAO1. However, when the mutant was grown in the presence of limiting rhamnose to deplete LptD, bacterial growth was slower, and the extracted lipid A gave peaks mainly attributable to lipid A species **1** with mono- or dihydroxylation and one or two AraN sugars (*m/z* 881.0/938.5/946.5; Figure 3, spectrum F, and Table S1). Thus, down-regula-

tion of *lptD* leads to the accumulation of lipid A forms that are not processed by PagL, as was the case for antibiotic-treated cells (Spectra B/C).

EM studies of the PAO1 P_{rhaB}*lptD* mutant

We reported earlier that *P. aeruginosa* PAO1 grown in MH broth with L27–11 revealed unusual internal accumulations of membrane-like material in thin sections by transmission EM.[1] The PAO1 P_{rhaB}*lptD* mutant examined in the same way after growth in LB medium revealed normal cell morphology for cells grown with high rhamnose (+*lptD*), but again internal accumulations of membrane-like material appeared in sections of cells grown with depleted LptD (−*lptD*; Figure 4A).

In scanning EM images, *P. aeruginosa* PAO1 cells grown in the presence of the antibiotic L27–11 appeared to have many external protrusions/blebbing of the OM (wt PAO1 with L27–11); these were not seen when cells were grown normally in MH broth (wt PAO1) (Figure 4B). A similar blebbing of the OM was also observed when the PAO1 P_{rhaB}*lptD* mutant was grown with depleted LptD (−*lptD*; Figure 4B), but not when *lptD* expression was induced with high rhamnose (+*lptD*). These studies revealed dramatic effects on membrane morphology by the actions of the antibiotic L27–11, similar to those caused by depletion of LptD.

Labelling LPS with [^{14}C]acetate

The effects of the antibiotic on LPS transport in cultures of *P. aeruginosa* were investigated by monitoring the incorporation of [^{14}C]acetate into LPS. [1-^{14}C]acetate was added to *P. aeruginosa* grown in MH broth. After 3 h, lipid A was extracted and analysed by TLC and autoradiography. TLC spots were identified in direct TLC-MALDI MS analyses: one major radioactive spot on the TLC plate from ^{14}C-labelled penta-acyl lipid A species **2** (Figure 5, lane 1). When [1-^{14}C]acetate was added to growing cells along with EDTA, the major labelled lipid A was the hexa-acyl form **3** (Figure 5, lane 2). This result is consistent with the known effect of EDTA on the OM. EDTA extracts metal ions and weakens interactions between LPS molecules, thereby allowing phospholipids into the outer leaflet, which in turn activates PagP.[14] When the antibiotic L27–11 and [1-^{14}C]acetate were added to growing cultures, the major ^{14}C-labelled lipid A observed was the ^{14}C-hexa-acyl form **1** containing two AraN sugar residues (Figure 5, lane 3). Only weak labelling of the normal penta-acyl form **2** and the hexa-acyl form **3** was observed in these experiments. This result demonstrates ^{14}C preferentially incorporates into LPS that has not been modified by PagL.

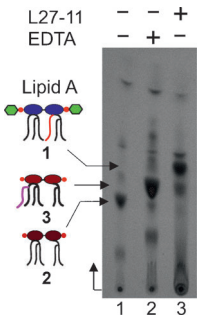

Figure 5. Autoradiograms showing ^{14}C-labelled lipid A from *P. aeruginosa* PAO1 grown in MH broth and separated by TLC (Supporting Information) after incorporation of [1-^{14}C]acetate. Lane 1: lipid A from PAO1 grown in MH broth; lane 2: lipid A from cells grown with addition of [1-^{14}C]-acetate and EDTA; lane-3: addition of [1-^{14}C]acetate, then antibiotic L27–11 (1 μg mL^{-1}), and growth for 2 h. The lipid A species **1**, **2** and **3** (Figure 1) are indicated.

Analysis of IM and OM components

The effects of the antibiotic on the membrane composition of *P. aeruginosa* PAO1 were analysed by sucrose density gradient analytical ultracentrifugation (Figure 6).[15] The lighter IM fractions (18–21) possessed the characteristic red colour of cytochromes and contained NADH oxidase activity, while the denser OM fractions (1–5) appeared translucent white; they also contained LptD, as detected by immunoblotting with anti-LptD antibodies, as in an earlier report.[15b] LPS was found concentrated largely in the heavy OM fraction. When cells were grown with sodium [1-^{14}C]acetate, two clear ^{14}C peaks were observed, one in the IM fractions of the gradient (19–22), and the other in the OM fractions (2–5; Figure 6).

When cells grown in MH broth with L27–11 (1 μg mL^{-1}) were harvested after 5 h ($OD_{600}\approx$0.8–1.0), a clear separation of IM and OM components was not observed; no distinct red and white translucent bands were apparent in the light and heavy fractions of the gradient; the total protein concentration and location of LptD were skewed towards lighter fractions, and a single peak of greatly reduced NADH oxidase activity appeared closer to the centre of the gradient; levels of incorporated ^{14}C were much higher than with untreated cells, and labelled LPS was skewed more towards the

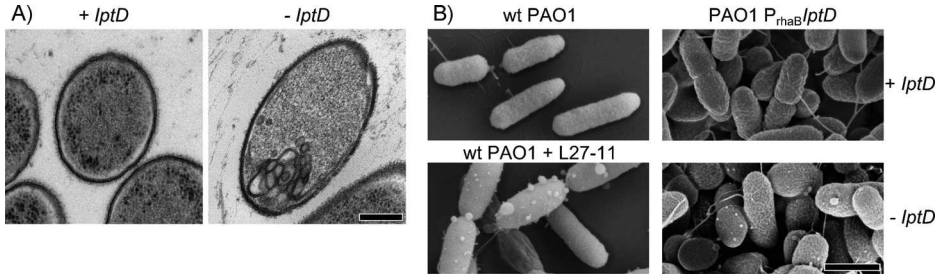

Figure 4. A) Transmission EM of sections of PAO1 P_{rhaB}*lptD* cells with LptD induced (+*lptD*) and depleted (-*lptD*), showing accumulation of membranous material in the latter (scale bar = 200 nm). B) Scanning EM of wt PAO1 grown with and without antibiotic L27–11 (left), and of PAO1 P_{rhaB}*lptD* mutant (right) with *lptD* induced (top) or depleted (bottom), showing blebbing of the membrane only when antibiotic is present, or *lptD* is depleted (scale bar = 1 μm).

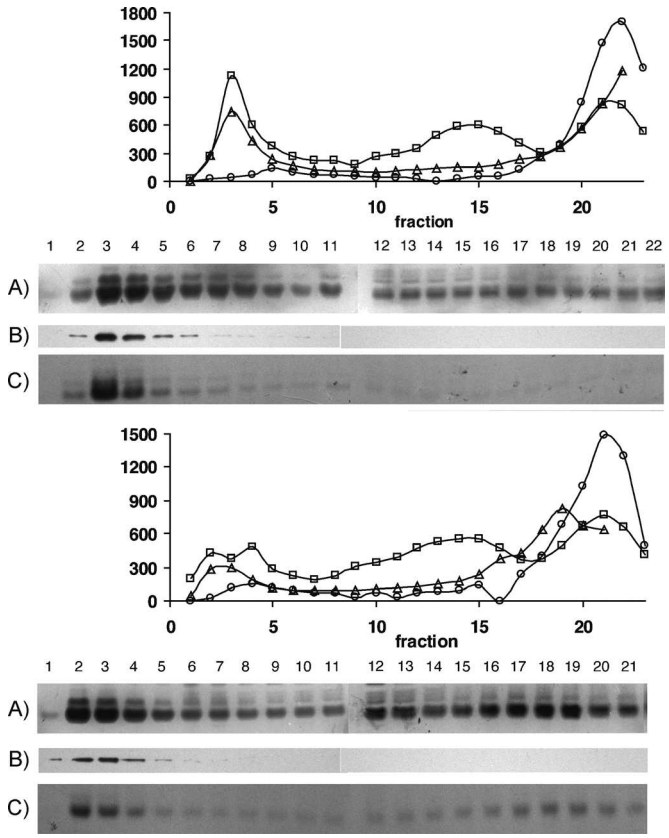

Figure 6. Top: Fractionation of IM and OM by sucrose gradient ultracentrifugation from *P. aeruginosa* PAO1 grown in MH broth with [1-^{14}C]acetate but without antibiotic. Bottom: Repeat of IM and OM fractionation 30 min after co-addition of [1-^{14}C]acetate and L27–11 [1 μg mL^{-1}] to cells grown in MH broth. Fractions (1–22) were taken from the bottom of the gradient and analysed for total protein content (□ [μg mL^{-1}]), NADH oxidase activity (○ [units ÷ 50 000 per 150 μL]) and incorporated radioactivity (△ [DPM × 20 per 50 μL]). A) LPS in SDS-PAGE detected by silver staining. B) LptD detected by immunoblotting. C) Radioactive LPS in SDS-PAGE gels detected by autoradiography.

lighter fractions. These results indicate that the IM and OM cannot be cleanly separated from cells treated in this way. However, when PAO1 cells were exposed to the antibiotic for a much shorter time (15–30 min), fractionation of the IM and OM components was still possible. A red band and NADH oxidase activity were then still concentrated in the IM fractions, and LptD was found in the OM fractions; however, more LPS was found associated with lighter (IM) fractions in the sucrose gradient (Figure 6, bottom). Also, when both [^{14}C]acetate and L27–11 were added to culture, ^{14}C-labelled LPS was skewed towards the lighter IM fractions in the sucrose gradient, compared to cells not exposed to the antibiotic, consistent with partial inhibition of LPS transport to the OM.

Discussion

LptD is an essential OM protein in *E. coli* that is required for the biogenesis of the cell envelope.[2a] Initial evidence for the role of LptD in OM assembly came from studies in *Neisseria meningitides*, where its deletion is tolerated and results in loss of LPS transport to the cell surface.[2b] LptD was later found to exist in a complex with the essential OM lipoprotein LptE in *E. coli*, where both proteins are required for LPS transport to the cell surface.[2c,e,16] All the Lpt proteins needed for LPS biosynthesis and transport from the cytoplasm in *E. coli* have now been identified, including LptA, which facilitates LPS transport across the periplasm and delivery to LptD/E in the OM.[2d,17]

We first set out to construct a conditional *lptD* mutant, which could be used to investigate the effects of depleting LptD in *P. aeruginosa*. LptD is essential for growth in *E. coli*, but not in *N. meningitides* or *Helicobacter pylori*.[2b,18] Attempts to create a knock-out mutant by insertional inactivation of *lptD* in *P. aeruginosa* PAO1 by means of a suicide vector containing internal fragments failed to provide viable mutants, thus suggesting that *lptD* is an essential gene in *P. aeruginosa*. A PAO1 conditional knock-out mutant was obtained by replacing the natural promoter of *lptD* in the chromosome with the rhamnose-inducible promoter P_{rhaB}.[12] This change, however, likely affects expression of the entire operon of overlapping *lptD-surA-pdxA* genes (Figure 2). The overlapping arrangement of *lptD* and *surA* is conserved in Gram-negative bacteria.[19]

SurA is a periplasmic chaperone that plays an important role in the assembly of the LptD/E complex in *E. coli*.[20] Strains lacking SurA exhibit defects that are indicative of OM perturbations,[21] and are hypersensitive to detergents and hydrophobic antibiotics.[22] Loss of SurA can be partly compensated for by other periplasmic chaperones such as Skp, but SurA plays an important role in folding of LptD in the OM in *E. coli*.[20] Hence, it was of interest to investigate whether loss of SurA has similar effects in *P. aeruginosa*. Growth of the conditional P_{rhaB}*lptD* mutant near to wt levels occurred only with high rhamnose in the medium, or with low rhamnose when the mutant was complemented in trans by *lptD*, thus showing that *lptD* is an essential gene in *P. aeruginosa*. However, under low rhamnose conditions, complementation of the mutant with both *lptD* and *surA* was required to restore wt levels of resistance to external detergent (SDS/EDTA). This is consistent with an important role for SurA in the assembly of LptD also in *P. aeruginosa*.[20]

The perturbation in membrane structure arising from depletion of LptD was visible by transmission EM as extensive folding into the cytoplasm of the IM (Figure 4A), perhaps caused by accumulation of LPS within the IM. Similar accumulations of "extra" membrane material were reported earlier within *E. coli* cells upon depletion of LptD and LptA,[2c,23] and in *P. aeruginosa* after treatment with antibiotic L27–11.[1] In addition, pronounced blebbing of the OM was seen in scanning EM images of both LptD-depleted and antibiotic-treated cells (Figure 4B). Blebbing and outer membrane vesicle formation in *P. aeruginosa* and *E. coli* are known to be stimulated by envelope stress.[24] The blebbing appears to occur randomly over the outer cell surface, and might conceivably be caused by a combination of factors, such as insertion of phospholipids into the outer leaflet, thereby resulting in weakened interactions between LPS molecules, which result in OM instability.

Four different approaches were followed to obtain evidence that the antibiotic L27–11 inhibits LptD-mediated LPS transport

to the OM in *P. aeruginosa*. First, modifications to the lipid A core of LPS induced during cell growth with the antibiotic, or after depletion of *lptD* in the conditional mutant, were analysed by ESI-MS. As mentioned in the introduction, several lipid A modification systems have been shown to exist in *P. aeruginosa*. The bacterium can modify LPS within the OM covalently by using enzymes PagL and PagP.[25] In addition, genes PA4512 and PA1936 in *P. aeruginosa* PAO1 encode homologues of LpxO, each of which likely α-hydroxylates one of the 2′- and 2-secondary acyl chains in lipid A.[25] Lipid A from PAO1 might, therefore, contain none, one or two α-hydroxy groups (X = H or OH, Figure 1C), depending upon the extent of turnover by these two IM hydroxylases. The major penta-acylated form of lipid A (**2**, mass of 1447) corresponds to lipid A that has undergone a single α-hydroxylation. A third type of modification to lipid A involves addition of the sugar AraN. *P. aeruginosa* contains numerous OM sensor kinases that allow cells to respond to various environmental signals, including low levels of divalent metal ions, the presence of cationic antimicrobial peptides and other causative agents of membrane stress. These include the PhoP/Q, PmrA/B and the ParR/S two-component regulatory systems that can up-regulate the expression of genes for the biosynthesis of AraN and its addition to the lipid A core of LPS.[26] For example, growth of PAO1 in low Mg^{2+} medium promotes addition of AraN to the 1 and/or 4′ phosphates of lipid A.[7d] In *E. coli* and *Salmonella typhimurium* an IM enzyme (ArnT) adds one or two AraN sugars to lipid A, most likely on the periplasmic side of the IM,[27] and a homologue of ArnT exists also in *P. aeruginosa* PAO1. It was shown earlier that modification of LPS with AraN can occur in combination with 3-O-deacylation by PagL in *P. aeruginosa*,[7d] unlike in *S. typhimurium*, where PagL is inhibited by modification of LPS with AraN.[28] Here, we showed that the enantiomer of L27–11 (ent-L27–11), which shows only weak antimicrobial activity with *P. aeruginosa* PAO1 (MIC 32 μg mL^{-1}), could lead to accumulation of LPS that had been modified by PagL and by addition of AraN (Figure 3D), thus confirming the finding of the earlier study: that modification with AraN can occur in combination with 3-O-deacylation by PagL.[7d]

Modifications catalysed by PagL and PagP in the OM, and by ArnT and LpxO1/2 in the IM, are therefore useful markers for LPS en route to the OM. The ESI-MS of lipid A measured under conditions that promote negative ion formation revealed that the most abundant form of lipid A isolated from PAO1 was the expected penta-acyl form (**2**, monohydroxylated, modified by PagL), although small amounts of the hexa-acyl form (**3**, monohydroxylated, modified by PagP) was also detected (Figure 3 and Table S1). After cell growth in the presence of antibiotic L27–11, the lipid A profile changed, with the rapid emergence of hexa-acyl lipid A forms (**1**) that are not modified by PagL or PagP, are twice hydroxylated and twice modified with addition of AraN. These changes are consistent with retention of increasing amounts of LPS in the IM when antibiotic is present.

Lipid A isolated from the conditional PAO1 P_{rhaB}*lptD* mutant grown with high rhamnose comprised the penta-acyl form (**2**, mono-hydroxylated), although small amounts of **2** with a single AraN units were also detected (Figure 3 and Table S1). Under low rhamnose conditions, however, the major lipid A detected by ESI-MS was the hexa-acyl form (**1**, dihydroxylated) with two AraN sugars (Figure 3 and Table S1). Thus, growth of *P. aeruginosa* PAO1 with antibiotic L27–11 leads to similar lipid A modifications as those that are observed in the conditional mutant when LptD is depleted. Complementary data were obtained by monitoring the incorporation of ^{14}C from [1-^{14}C]acetate into LPS in the presence of antibiotic (Figure 5). Under normal growth conditions (without antibiotic), formation of mainly ^{14}C-lipid A **2** was observed, while with EDTA present, mainly the ^{14}C-palmitoylated lipid A form **3** was observed, because of the action of PagP. However, with antibiotic present, the profile of modified ^{14}C-lipid A changed, with accumulation of the ^{14}C-hexa-acyl form **1** that contains two AraN sugars.

Transport of LPS was also investigated by separating the IM and OM by sucrose gradient utracentrifugation. However, when PAO1 cells were exposed to L27–11, it quickly became impossible to obtain clear separation of IM and OM components, most likely attributable to major concomitant changes in membrane structure and morphology, as detected by EM. Similar observations have been made when LptD, LptA or LptB was depleted in *E. coli*.[17b,23] This further underscores the similar effects elicited by the antibiotic and by depletion of LptD. Nevertheless, by exposing PAO1 cells to the antibiotic for only short times, fractionation of the IM and OM components was still possible, and under these conditions a trend was apparent, with a skewing of LPS (including ^{14}C-labelled LPS de novo biosynthesised from [1-^{14}C]-acetate) from the OM fractions towards the IM (Figure 6).

The results reported here are not consistent with LptD acting merely to transport L27–11 across the OM, but rather support a mechanism of action for the antibiotic that involves inhibition of LptD-mediated LPS transport to the OM. Many details of this mechanism of action remain to be elucidated, not least how and where the antibiotic binds to the folded LptD protein, and how this binding event is linked to inhibition of LPS transport. LptD plays an essential role in OM biogenesis in many Gram-negative bacteria. The peptidomimetics act selectively against *Pseudomonas* sp., perhaps depending on sequence and/or structural differences in LptD between different Gram-negative bacteria. So far the 3D structure and mode of action of LptD are unknown. However, the conserved domain organisation and exposed location in the OM suggest that LptD might nevertheless be an interesting target in screening efforts to discover new drugs active against other Gram-negative pathogens. Available inhibitors of LptD, such as L27–11, might also serve as molecular tools for more detailed mechanistic studies of LPS transport. Finally, information on the mechanism of action of these antibiotics is crucial for their clinical development. The first clinical candidate, POL7080, has already been identified and has now entered a phase I human clinical trial.

Experimental Section

Bacterial strains and plasmids: Bacterial strains, plasmids and oligonucleotides are described in the Supporting Information.

Construction and complementation of the P_{rhaB}*lptD* mutant: A fragment (~300 bp) spanning the 5′ region of *lptD* was amplified by PCR with primers lptDcm-F and lptDcm-R, homologous to regions around, and downstream of, the start codon. The PCR product was digested with NdeI and XbaI and cloned into XbaI-digested pSC200Gm,[29] downstream of P_{rhaB} (Figure 2B). The resulting plasmid was introduced into *E. coli* CC118 λ pir with selection on gentamicin (Gm; 25 μg mL^{-1}) and glucose (0.5%). The plasmid was then transferred into *P. aeruginosa* PAO1 by triparental mating, with selection of the conditional mutant on PIA (Difco) medium supplemented with rhamnose (0.5%) and Gm (150 μg mL^{-1}). The primer pair test1/test2 was used to confirm by PCR that the plasmid had integrated at the expected site in the chromosome (see Figure 2B and Figure S1). The methods used to complement the P_{rhaB}*lptD* mutant with plasmid-borne copies of *lptD* or overlapping *lptD* and *surA* are given in the SI.

Growth and viability of the P_{rhaB}*lptD* mutant: Growth characteristics and viability of the mutant, including Live/Dead staining and susceptibility to SDS/EDTA, are shown in Figures S2 and S3. The mutant was unable to grow with glucose on agar plates or in liquid medium. Growth was weak in the presence of low rhamnose (0.001% (*w/v*)), but in liquid medium and agar with high rhamnose (0.01%), growth occurred as for the wt strain (Figure S2).

Isolation and analysis of lipid A: LPS was isolated from *P. aeruginosa* by the hot phenol–water extraction method.[30] Lipid A was prepared from LPS by hydrolysis in sodium acetate (pH 4.5), and then isolated using the method of Bligh–Dyer.[31] Briefly, cells from culture broth (40 mL, OD_{600} = 1.0) were harvested by centrifugation (4 °C, 3500 *g*) and washed twice with phosphate-buffered saline (PBS; 5 mL, NaH_2PO_4 (2.5 mM), Na_2HPO_4 (7.5 mM), NaCl (145 mM), pH 7.2). The pellet was resuspended in PBS (0.8 mL, pH 7.2) and a mixture of MeOH (2 mL) and $CHCl_3$ (1 mL) was added. After 60 min the insoluble material was collected by centrifugation (4 °C, 3500 *g*). The pellet was washed with $CHCl_3$/MeOH/H_2O (1:2:0.8 (*v/v/v*), 5 mL), and suspended in sodium acetate buffer (1.8 mL, 12.5 mM, pH 4.5), containing SDS (1% (*w/v*)) with agitation. The mixture was heated in a boiling water bath for 30 min to cleave the glycosidic linkage between Kdo and lipid A. MeOH (2 mL) and $CHCl_3$ (2 mL) were added to the hydrolysed material, and, after centrifugation (5 min, 200 *g*), the lower phase (mainly $CHCl_3$) was collected and washed twice with $CHCl_3$/MeOH/H_2O (2:2:1.8, *v/v/v*, 4 mL). The washed lower phase was dried under a stream of N_2 and stored at −20 °C. Details of the TLC separation of lipid A and conditions for analysis by MS are provided in the Supporting Information.

Membrane fractionation by ultracentrifugation: Fractionation of IM and OM components by sucrose gradient ultracentrifugation was as described elsewhere.[15b] Fractions taken from the bottom of the sucrose gradient were analysed for NADH oxidation activity as described;[32] protein concentrations were determined by using a Pierce BCA protein assay kit (Thermo Scientific); LPS was analysed by 15% SDS-PAGE in Tris-HCl buffer (0.1 M, pH 6.8) containing SDS (2%), sucrose (20%), 2-mercaptoethanol (1%) and bromophenol blue (0.001%); LPS was detected in gels by silver staining;[33] LptD was detected by immunoblotting with anti-LptD polyclonal antibodies as described.[1]

Electron microscopy: The methods used to prepare samples for EM were as previously described,[1] and in the Supporting Information.

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Keywords: antibiotics · inhibitors · lipids · lipopolysaccharide · phospholipids

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